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(54) Title: FIELD SYNTHESIS AND OPTICAL SUBSECTIONING FOR STANDING WAVE MICROSCOPY

(57) Abstract

In an improved optical microscope for observing a luminescent specimen, the specimen is excited by a time-multiplexed series of standing wave fields. Then an image of the specimen is recorded and displayed. This specimen can be incrementally moved and additional images can be created and combined. Images of the specimen can also be created when there are nodes or antinodes at the focal plane of the microscope. These images can also be combined to produce an improved image of the specimen.

- 1 -

TITLE

FIELD SYNTHESIS AND OPTICAL SUBSECTIONING
FOR STANDING WAVE MICROSCOPY

Background of the Invention

The invention relates to luminescence optical
microscopy and particularly to an apparatus and method
for selectively optically illuminating particular zones
in a specimen such as a biological cell or tissue.

Field of Invention

Optical microscopy using fluorescence tagging
for the determination of three-dimensional structure of
cells and tissues is an important diagnostic and research
procedure. There are a great number of dyes which can be
attached to various structures within the cell. When
excited by a particular wave length of light these dyes
will fluoresce or phosphoresce. For example, several
common dyes will emit a red glow when excited by green
light. Hence, one can see structures to which a
fluorescent dye is attached. The presence and location
of the tagged structures can provide important diagnostic
and structural information for basic research and
clinical diagnostics.

Fluorescent imaging, in particular, is of vast
utility in cell biology because of the high specificity
of fluorescently labeled protein analogs, antibodies,

- 2 -

hybridization probes, enzyme substrates, lipid analogs,

and peptides, as well as stains. Fluorescence

micrographs of extremely complicated objects such as

intact cells typically show clearly the distribution of

the tracer molecules, all other components being

"invisible". The important optical characteristics of

many biological specimens that allow for this simple

interpretation of the image field is that cells are

generally weakly refractive and weakly absorptive

objects.

Conventional microscope images contain

information about the 3-D structure of the object when

the depth of field of the lens system is smaller than the

axial dimension of the specimen. This means that in a

single image, the axial location of a particular feature

is encoded by its degree of defocus. A 3-D image data

set, which is a "zero-order" estimate of the true

structure of the object, is obtained by recording a

series of images as the object is stepped through the

focal plane of the microscope, a procedure known as

optical sectioning microscopy (OSM). Each image is a

spatially filtered axial projection of the object, and

each generally contains in-focus and out-of-focus

features. One of the central problems in 3-D microscopy

is the removal of out-of-focus features from the 3-D

image by optical and image processing methods thereby

deriving a refined estimate of the true object.

- 3 -

In fluorescence microscopy there is a linear
relationship between the emitter distribution in the
object and the intensity distribution in the image field.
This is caused by the mutual incoherence of fluorescence
emission. Dye molecules in the specimen radiate
independently so that the individual intensity fields are
simply superimposed in the image plane. It is possible
to deconvolve the 3-D image to eliminate the out-of-focus
portions. However, for the various methods that have
been proposed there is a trade-off of recovery of high
frequency structure for accuracy or stability.

The alternative to computational refinement of
optical sectioning microscopy (OSM) is confocal scanning
fluorescence microscopy (CSFM) in which direct optical
spatial filtering is used to remove out-of-focus light
waves from the detector field. In one version of this
type of instrument fluorescence is excited in the
specimen by a highly focused beam. In the image plane of
the microscope a pin hole is placed at the point
optically conjugate to the focal point of the beam and a
high gain, low noise detector is placed behind the pin
hole. The microscope acts as a spatial filter that
detects efficiently only fluorescence photons that
originate near the beam focus. 3-D image data is
obtained by a raster scanning of the beam relative to the
specimen, either optically or mechanically, and stepping

- 4 -

the specimen axially through the focal plane to get
stacked images. Confocal methods have several
shortcomings. For example, such images often have a low
signal to noise ratio. Hence, the resolution of the
image is often severely compromised. Also, scanning
usually is relatively slow, with scan times up to 64
seconds per frame for high signal-to-noise images.
Indeed, there are many circumstances in which this
technique cannot be utilized.

For fundamental reasons, a fluorescence
microscope is more severely limited in axial (depth, or
inter-image plane) resolution, as opposed to transverse
(in the image plane) resolution. Consider a microscope
with a lens having a high numerical aperture (NA) and a
specimen of refractive index n illuminated by a light
beam having a wavelength λ. The well-known Rayleigh
resolution formula, $0.61\lambda/(NA)$, sets transverse
resolution at about 0.2 μm via direct imaging. This can
be halved, in principle, by confocal scanning. In
comparison, the axial equivalent of the Rayleigh formula,
$2n\lambda/(NA)^2$, is in the range 0.7-0.9 μm, typical for high-
quality fluorescence OSM image sets. Computational image

- 5 -

processing or confocal scanning can reduce this to
0.4-0.5 μm. A more restrictive analysis, the Rayleigh
quarter-wave criterion, $\lambda/8n \sin^2(1/2 \sin^{-1} NA/n)$, gives
a theoretical axial resolution in the range 0.13-0.17 μm
for the best microscope lenses. This has been
demonstrated in transmitted light microscopy, but not in
fluorescence, due to the lack of mutual coherence in
fluorescence imaging, and the generally lower signal-to-
noise level. Therefore when the specimen contains fine
stratified structural features, or simply when it is
thinner than the depth-of-field, fluorescence OSM or even
CSFM is unable to yield significant 3D information.

United States Patent No. 4,621,911 discloses a
method and apparatus called standing wave luminescence or
fluorescence microscopy (SWFM) in which a specimen is
illuminated in a fluorescence microscope by means of a
standing wave field at the excitation wave length. This
field is preferably produced by crossing two equal
amplitude coherent beams from a laser. The direction of
the beams is such that the nodal and anti-nodal planes in
this field are parallel to the object plane of the
microscope. Under this condition fluorescence is excited
in laminar zones in the specimen, maximally at the
location of each anti-nodal plane. One of these planes

- 6 -

can be made coincident with the in-focus plane. In this way in-focus features of the specimen are made brightly fluorescent. Immediately adjacent out-of-focus features above and below the in focus plane are in nodal zones and are, therefore, only weakly fluorescent.

United States Patent No. 4,621,911 teaches a theory and embodiments for creation and manipulation of a periodic standing wave field superimposed with the specimen in a fluorescence microscope, and that sets of images obtained by standing wave excitation contain Fourier coefficient information on the axial (depth) structure of the object down to an axial resolution limit of $\lambda/4n$, as small as 0.068 μm. The embodiments of the patent include several methods for producing a standing wave field by crossing two equal-amplitude collimated s-polarized coherent beams at complementary angles relative to the axis of the microscope. These embodiments include the use of total internal reflection (TIR), a mirror or prism, or a wavelength-selective high reflector to fold a laser beam in the specimen region of the microscope, independent coherent beams entering the specimen from opposite sides, or a re-entrant beam that first emerges from the objective lens into the specimen. The patent also includes embodiments where the nodal planes are not parallel to the object focal plane although the parallel

condition is of principal interest here. Nevertheless,
the method and apparatus of the '911 patent do not
overcome the problems associated with the presence of out
of focus luminescent portions in the image.

Summary of the Invention

We provide improved methods and apparatus which
overcome the out of focus problems and clearly display
the transverse and axial position of luminescent
structures in the specimen. As in the apparatus of
Patent 4,621,911, a fluorescence microscope is equipped
with an optical system for standing wave excitation of
the specimen. In a first present preferred method we
manipulate a single standing wave field to show the axial
structure of a specimen, even when the specimen is so
thin that it is entirely within the depth-of-field of a
high resolution microscope. We call this extension
optical subsectioning, and have found a practical axial
resolution limit of $\lambda/8n$ (approximately 0.05 μm) in thin
specimens. In a second present preferred embodiment a
nonperiodic excitation field is generated in the specimen
such that the excitation intensity is peaked only at the
object focal plane. In this case, intersecting beams
entering the specimen from opposite sides are swept in
angle to generate a continuous series of standing wave
fields that differ in node spacing, but all with an

- 8 -

antinode at the object focal plane. A single image is
recorded with the swept excitation, with the net result
of the time-multiplexing being the preferential
excitation of structures in the specimen that lie in the
in-focus plane. This differs from the method of the '911
patent in that the net excitation field is peaked only at
the in-focus plane, and not at evenly-spaced antinodal
planes. We call this method excitation field synthesis
(EFS) or field synthesis fluorescence microscopy (FSFM).
It represents an extension of standing wave microscopy
theory and practice to the high-aperture limit, where the
depth-of-field of the optics is less than the axial
dimension of the specimen.

In our method and apparatus we direct two beams,
which can be a single beam reflected back through a
specimen, to create distinct nodes and anti-nodes such as
is taught in U.S. Patent No. 4,621,911. We prefer to
direct the beams through the lens of the microscope. In
one embodiment the beam reflects directly back from a
mirror positioned underneath the specimen.
Alternatively, we use an active phase conjugator to
generate the return or re-entrant beam in a standing wave
illuminator. The phase conjugator generates the time-
reversed version of the excitation beam that first passes
through the specimen, so that a standing wave field of

high spatial modulation is created, even in specimens
where the incoming planar wavefronts have been aberrated
due to refractive index inhomogeneties. In another
embodiment we use a beam splitter to create two beams
from a single light source. The two beams preferably are
directed to enter the specimen from opposite sides.

In yet another embodiment the specimen is
illuminated in a manner previously discussed and an image
of the specimen is recorded in a camera and stored. Then
the specimen is moved a selected axial distance. Again
the specimen is illuminated and a second image is created
and stored. This process can be continued until a
desired number of images of the specimen are created.
These images are then combined by image proccessing to
produce one or more combined images of the specimen.

Other objects and advantages of the invention
will become apparent from a description of the preferred
embodiments shown in the figures.

Brief Description of the Figures

Figure 1 is a schematic representation of the
standing wave illumination of the prior art wherein a
specimen is in a standing wave field of s-polarized light
and on the optical axis of a microscope.

Figure 2 is a schematic representation
illustrating the formation of a standing wave field by

total internal reflection of an incident beam from a
cover glass.

Figure 3 is a side view of a specimen placed in
the object focal plane of a microscope.

Figure 4 is a diagram showing a first present
preferred embodiment of our improved standing wave
microscope.

Figure 5 is an enlarged view of the specimen
being illuminated in the microscope of Figure 4.

Figure 6 is a block diagram of a image processor
used with the microscope of Figure 4.

Figure 7 is a schematic diagram of the second
preferred embodiment of our microscope.

Figure 8 is a graph of overlapping light beams
having different nodal spacings.

Figure 9 is a graph illustrating movement of the
nodal plane for optical subsectioning.

Principles of Operation

In a standing-wave microscope, two plane-wave
fields from a laser are crossed at complementary angles
in the specimen volume, where they interfere (Fig. 1).
When the two fields are s-polarized and of equal
amplitude, the resulting interference pattern has an
electric field intensity that varies only axially, as

- 11 -

$$I_{ex}(z) = I_0 [1 + \cos (Kz + \Phi)]$$

where $K=(4\pi n/\lambda)\cos\theta$, λ is the wavelength and n is the specimen refractive index. Fluorescence is excited in the specimen in proportion to $I_{ex}(z)$. The nodes or antinodes, which are planes parallel to the focal plane, have a spacing $\Delta s = \lambda/2n \cos\theta$. By controlling the angle θ, the node spacing can be varied down to a minimum value of $\lambda/2n$, when the two beams are counterpropagating along the axis of the microscope. By shifting the phase of one of the beams, the relative position of the antinodes within the specimen can be changed, at constant node spacing.

It is straightforward to estimate the axial resolution in SWFM. Two particles which are separated axially by half the node spacing can be illuminated alternately by shifting the phase of the standing wave pattern. With blue light excitation and a specimen refractive index of 1.33 (water) or higher, $\lambda/4n$ is 0.09 μm or less. In practice, it has been possible to resolve

- 12 -

particles separated axially by a quarter fringe (0.045

μm) in specimens where there is little overlapping

structure. Because these distances are considerably less

than the depth-of-field normally obtained in fluorescence

microscopy, "optical subsectioning" is possible. That

is, in thin specimens that fall entirely within the

depth-of-field of a high numerical aperture (NA)

objective lens, axial structure can be observed purely by

calibrated movement of an antinodal or nodal plane

through the specimen, with no mechanical refocusing

required. Even when the specimen is thick, discrete

outlying structure may be discriminated by degree of

defocus from in-focus features, so that optical

subsectioning will still be useful. In mathematical

terms, standing wave excitation is equivalent to axial

modulation of the point spread function (PSF) which in

turn is equivalent to shifting the optical transfer

function (OTF) axially in reciprocal space by a distance

equal to the spatial frequency of the standing wave

field. This permits recovery of spatial frequency

information that is absent or very heavily attenuated

under incoherent excitation.

Optical subsectioning is a subtractive process

in which a single standing wave field is manipulated to

resolve the relative axial position of two or more

stratified structures in a thin fluorescently labeled

- 13 -

specimen. A thin specimen is one which lies entirely

within the depth-of-field of the microscope and,

strictly, within one node period of the standing wave

field, 0.17 μm under typical conditions. In practice,

the phase of the field is adjusted so that a nodal plane

is coincident with one stratum. The structural features

of that stratum then fluoresce only weakly, and an image

is recorded that shows primarily the structures in other

strata, i.e., a subtractive image. The nodal plane is

then moved by a known distance to null the fluorescence

from a neighboring stratum, and a complementary image is

then recorded. In the simplest case, two structures that

overlap in a conventional fluorescence image will each

appear distinctly in two standing wave images, where a

nodal plane is located first at one structure, and then

the other.

A standing-wave microscope is, in fact, a type

of interferometer in which the dye molecules in the

specimen act as the primary detectors of the excitation

field. In our original design (Fig. 2), total internal

reflection was utilized to fold a collimated laser beam

at the specimen cover glass to form the periodic field,

which made the use of a high numerical aperture (NA)

immersion lens difficult. This problem is solved by use

of low-divergence gaussian beams propagating within the

aperture of a high-NA lens, giving maximum resolution and

- 14 -

light collection efficiency. In the simplest

configuration (Figs 4 and 5), the beam 21 emerges from

the lens 8, passes through the specimen 2, and is back-

reflected by a closely-apposed mirror 16. One could use

a phase conjugator in place of a mirror 16. The mirror

is moved axially by a piezoelectric drive 14, which

causes an equal axial shift of the excitation field

planes through the specimen. The optics are adjusted so

that the gaussian beam exiting the objective 8 contracts

slightly to a large-diameter waist (150 μm) at the

mirror, 0.1-0.2 mm beyond the specimen. The standing

wave field is then due to the superposition of the

gaussian field and its reflection. In this condition,

the nodal surfaces of the unperturbed standing wave

field, although curved in principle, are flat to better

than 1 part 20,000 over the field of view. Therefore,

these surfaces are called nodal planes. Nodal planes are

always parallel to the mirror in this system.

A more versatile optical system having a laser

64 for a light source was also designed, with objective

lenses 8 and 55 positioned on opposite sides of the

specimen (Fig. 7). A prism splitter 56 is used to

amplitude-divide the expanded beam, so that a low

divergence gaussian enters the specimen 2 independently

from each side. Since the coherence length of a 1 m ion

laser is only about 30 mm, the two beam paths in the

- 15 -

microscope are typically matched to within 5 mm. A
piezoelectric drive 74 on a mirror 72, 73, 74 in one beam
path serves to adjust the phase, in this case mirror
movement being a full wavelength per cycle of the
standing wave field. One advantage of the two beam
system is that unaberrated wavefronts enter the specimen
on both sides, compared to the mirror system where phase
errors accumulate on both passes when the specimen
refractive index is heterogeneous. The second advantage
of this configuration is that by sweeping the beam angle
(θ) in the specimen, standing wave fields of different
spatial period can be time-multiplexed in the specimen
during the acquisition of a single image. The advantage
of this is described below.

The two-beam system provides the means for
excitation field synthesis (EFS). The microscope
operates like an OSM system, in that fluorescence images
of the specimen are recorded in an electronic camera as
the specimen is stepped through the focal plane. It
differs from OSM in that for each image, the specimen
will be excited by a continuous sequence of standing wave
fields that differ in axial node spacing, but all with an
antinode located at the focal plane of the microscope.
The sequence of fields is generated by sweeping the beam
crossing angle during excitation of the specimen. For
every field in the sequence, fluorescence will be excited

- 16 -

maximally at the focal plane. Away from this plane, the

phases of each field differ, so that the excitation

intensity averaged over all fields in the sequence will

be less. Optionally, a second fluorescence image can

then be recorded with the same sequence of standing wave

fields, except that each is adjusted to have a node at

the focal plane. In this case, fluorescence will be

· excited minimally at the focal plane, but at similar

averaged levels away from it. Digital pixel-by-pixel

subtraction of the nodal image from the antinodal image

gives a result for which the effective excitation field

is peaked at the geometric focal plane, but decays to

zero above and below this plane.

In practice, the sequence of standing wave

fields can be generated by sweeping the beam-crossing

angle θ through the full range accessible within the

aperture of the objective lens and condenser. For a

water-immersion system having a numerical aperture of

1.2, for example, the range is $\pm64°$. The synthesized

field for the antinodal image will then be

- 17 -

$$I_{EFS}(Z) = \int_{-\theta_m}^{\theta_m} \frac{1}{2}\left\{1 + \cos[K_o z \cos \theta]\right\} d\theta$$

$$= \theta_m(1 + J_o(K_o z)) + \sum_{n=1}^{\infty} (-1)^{n-1} \frac{\sin 2n\,\theta_m}{n} J_{2n}(K_o z)$$

$$= 1.12\,(1 + J_o(K_o z)) + 0.78\,J_2(K_o z) + 0.49\,J_4(K_o z)$$
$$+ 0.15\,J_6(K_o z) - 0.10\,J_8(K_o z) - 0.19\,J_{10}(K_o z)$$
$$- 0.13\,J_{12}(K_o z) - 0.01\,J_{14}(K_o z) + \cdots$$

where $K_o = 4\pi n/\lambda$. For the nodal image, only the constant
term does not change sign, so that the difference image
is weighted by the sum of the Bessel terms. This sum is
peaked at the focal plane, and decays to zero in an
oscillatory manner above and below it (Fig. 8). For an
idealized optical system in which the numerical aperture
is equal to the specimen refractive index (NA = n, θm =
90°), the weighting of the difference image is simply
$J_o(K_o z)$.

The effect of field synthesis on the OTF can be
illustrated directly. Fourier transformation of the
above equation gives

$$I_{EFS}(k_z) = \begin{cases} 2\pi\theta_m\delta(k_z), & |k_z| < K_o\cos\theta_m \\ \pi \Big/ \sqrt{K_o^2 - k_z^2}, & K_o\cos\theta_m < |k_z| < K_o \\ 0, & |k_z| > K_o \end{cases}$$

which is a piecewise discontinuous function of axial
spatial frequency. $I_{EFS}(k_z)$ is convolved with the OSM

- 18 -

OTF to give the EFS OTF. Therefore, it can be seen that

for an EFS system operating within the aperture of

existing high-NA objective lenses, the OSM OTF gets

expanded piecewise into an extended axial spatial

frequency band. The EFS OTF has the same transverse

limit as in OSM, but is significantly extended axially.

Purely axial wave vectors with frequencies in the range

of $K_o\cos\theta_m$ to K_o are recovered directly, and the bandpass

limit extends beyond this for near-axial wave vectors.

Description of the Preferred Embodiments

The present invention utilizes intersecting

beams to create a standing wave pattern. Several methods

for creating such a pattern are described in United

States Patent No. 4,621,911. Figure 1, which was taken

from that patent, shows the intersection at a fluorescent

or phosphorescent specimen of two coherent, collimated,

monochromatic beams of light, A and B, of a wavelength

suitable for excitation of fluorescence or

phosphorescence in the specimen. Rays A represent the

propagation of a collimated beam (plane waves) that makes

an angle θ with the optical axis of the microscope. Rays

B represent the propagation of a second collimated beam

that makes an angle $180° - \theta$ with the optical axis, and

is coplanar with A and the optical axis.

- 19 -

It is also possible to create a standing wave
pattern using reflection so that the reflected beam
intersects the incident beam. This technique is
illustrated in Figure 2. A sufficiently coherent light
source (not shown) directs a collimated beam C which
passes through the specimen 2. That beam strikes the
cover glass 4 and is reflected as collimated beam D.
Since beams C and D are s-polarized and intersect, a
standing wave pattern 6, indicated by dotted lines, is
formed. The microscope lens 8 is positioned so that its
focal point 9 in focal plane 10 is within the standing
wave pattern in the specimen.

When both beams A and B or C and D make the same
angle θ with respect to the optical axis of the
microscope, as shown in Figures 1 and 2, the antinodal
and nodal planes of the standing waves are parallel to
the focal plane. Therefore, fluorescence will be excited
in the specimen in laminar zones that show the axial
structure of the object. This can be most clearly seen
in Figure 3.

A side view of the specimen 2 mounted on a glass
slide 6 is shown in Figure 3. The specimen 2 is under a
cover glass 4. The microscope lens 8 is positioned so
that the object focal plane 10 is within the specimen 2.
If the specimen is illuminated in the manner shown in
Figures 1 or 2 a series of laminar zones 12 will be

- 20 -

created within the specimen. The node spacing (ΔS) of
the excitation field varies with changes in the
wavelength (λ) of the beam and its angle (θ) relative to
the optical axis 3. That is, (ΔS) = $\lambda/2n \cos \theta$ which is
a minimum of $\lambda/2n$ at $\theta = 0°$. The relative position of
the nodes and the specimen can be varied at constant node
spacing by shifting the phase of one of the beams. In
practice, total internal reflection was utilized to fold
one beam at the cover glass 4 so as to set up a standing
wave as shown in Figure 2. In the method of Figure 5, θ
is limited by the lens system to a maximum value of θ_m =
$\sin^{-1} NA/n'$. In the system of Figure 2, θ is limited by
a minimum value equal to the critical angle at the cover
glass 4.

Many cells can be tagged with a dye that is
excited by green light to fluoresce red; so we can use a
green laser for our light source. For other dyes we may
also use blue, red, yellow and even ultraviolet lasers.
As shown in Figure 4 it is also possible to use an
incoherent light source 20 such as a high pressure
mercury lamp with a beam collimator 19, polarizer 22 and
bandpass excitation filter 23. For the microscope 18
shown in Figure 4 the imaging system is comprised of

- 21 -

lenses 8 and 28, dichroic reflector 26, emission filter
27, beam splitter 25, eyepiece 24, camera 30, image
processor 32 and display 34. Light source 20 emits a
light beam 21 which is expanded s-polarized and
collimated. Then the beam is reflected by dichroic
reflector 26. Green light passes through lenses 28 and
8, cover glass 4 and specimen 2 until it is reflected by
mirror or phase conjugator 16. This causes a standing
wave pattern shown in Figure 5 to be created in the
specimen. The incident and reflected green light beams
also cause luminescent tags within the specimen to emit
red light 29. Emitted red light passes from the specimen
through lenses 8 and 28 and through dichroic filter 26
and emission filter 27 and is directed by beam splitter
or reflector 25 to eyepiece 24 or camera 30 or both. An
electronic camera 30 with image processor 32 and display
34 records and displays an image. The display could be a
cathode ray tube or film.

Referring to Figure 5, the incident beam 21 of
green light passes through the cover glass and specimen
and is reflected by reflective surface 17 of mirror 16 as
reflected beam 31. The intersecting incident beam 21 and
reflected beam 31 create a standing wave pattern within
the specimen. Furthermore, this light causes luminescent
tag 36 within the specimen 2 to emit red light indicated
by beam 26. The red light 26 is then directed to the

- 22 -

camera image processing unit and display as shown in
Figure 4. The optics are adjusted so that a gaussian
beam 21 exiting the objective 8 contracts slightly to a
large-diameter waist (150μm) at the reflective surface 17
of the mirror 16 which is preferably 0.1-0.2 mm beyond
the specimen 2. The standing wave field is then created
by the superposition of the gaussian field and its
reflection. In this condition, the nodal surfaces of he
unperturbed standing wave field, although curved in
principle, are flat to better than 1 part in 20,000 over
the field of view.

The image which is created from the emission of
light 29 by the luminescent tag 36 can be enhanced using
known image processing technology. That image can be
further improved using the methods described herein.
Therefore, the image processor 32 should include a
central processing unit 37, read only memory 38, and a
random access memory 39 as indicated by Figure 6.
Normally, the image will be digitized using an A/D
converter 40 in the image processor 32 or in the camera
30. The digital image is enhanced by the central
processing unit 37 according to a program in memory 38.
Both the original and enhanced images can be stored in
memory 39. The enhanced image is converted to analog
form by D/A converter 41 for display.

- 23 -

A more versatile optical system with objective
lenses positioned on opposite sides of the specimen is
diagrammed in Figure 7. A laser emits a light beam 65
which passes through beam expander 66 and lens 67 to
pivotable mirror 68 and a scan system 69 for field
synthesis. The scan system 69 contains a movable scan
mirror 68 and telescope 58. The beam 65 is split by beam
splitter 56. A portion of the light is directed by
mirrors 71, 72 and 73 through phase control 52, tube lens
53 and objective 55 through the specimen 2. A second
portion of the beam is directed by prism 54, lens 22,
dichroic reflector 26 and objective lens 8 through the
specimen 2. Light 29 emitted from the specimen 2 passes
to camera 30 or oculars 42 through lens 8, dichroic
reflector 26, barrier filter 46 and tube lens 44. The
beam splitter and prism are used to amplitude-divide the
expanded gaussian beam, so that nearly flat wave fronts
enter the specimen independently from each side. Since
the coherence length of a 1 m laser is only about 30 mm,
the two beam paths in the microscope are matched to
within 5 mm. A piezoelectric drive 74 can be provided on
a mirror 71, 72, 73 or dichroic reflector 26 in one beam
path to adjust the phase. In this case mirror movement
is a full wavelength per cycle of the standing wave
field. One advantage of the two beam system is that
unaberrated wavefronts enter the specimen on both sides,

- 24 -

compared to the mirror system of Figure 5 where phase

errors accumulate on both passes when the specimen

refractive index is heterogeneous. The second advantage

of the configuration of Figure 7 is that by sweeping the

beam angle (θ) in the specimen, such as by rotation of

mirror 68, standing wave fields of different spatial

period can be time-multiplexed in the specimen during

acquisition of a single image. If this is done while

keeping an antinode fixed at the object focal plane,

sweeping provides a means for synthesizing a nonperiodic

excitation field peaked at the in-focus plane of the

specimen.

The devices shown in Figures 4 thru 7 are

particularly useful for two methods of specimen imaging.

In one method, the specimen 2 is excited by a time-

multiplexed sequence of standing wave fields that differ

in axial node spacing. This generates a set of wave

patterns which if superimposed would look like the upper

graph of Figure 8. There three waves 75, 76, 77

indicated by solid, dotted and chain lines are shown. By

superimposing a set of standing wave fields having

different node spacing, but all having an antinode at the

focal plane in the specimen an effective field that is

peaked at the focal plane can be synthesized.

Therefore, for every field in the sequence,

fluorescence will be excited maximally at the focal

plane. Away from this plane, the phases of each field
differ, so that the excitation intensity averaged over
all fields in the sequence will be less. Optionally, a
second fluorescence image can then be recorded with the
same sequence of standing wave fields, except that each
is adjusted to have a node at the focal plane. In this
case, fluorescence will be excited minimally at the focal
plane, but at similar averaged levels away from it.
Digital pixel-by-pixel subtraction of the nodal image
from the antinodal image gives a result for which the
effective excitation field is peaked at the geometric
focal planes, but decays at zero above and below the
plane. The sum of a series of curves, such as the curves
75, 76 and 77, is shown in the bottom graph of Figure 8.
In practice, the sequence of standing wave fields can be
generated by sweeping the beam-crossing angle θ through
the full range accessible within the aperture of the
objective lens and condenser by use of scan mirror 68.

Figure 9 illustrates optical subsectioning. A
specimen 2 has tagged objects a and b. In the first case
90, a nodal plane is made coincident with object "a", so
that the image will show the fluorescence of "b". In the
second case 92, the node has been shifted to the axial
location of "b", so that a second image will then show
"a". The process can be easily extended to three or more

- 26 -

close stratified objects, with the result that a series

of images are obtained which contain linear combinations

of contributions from each stratum. Linear digital

processing can then be used to extract images

corresponding to each stratum.

Two problems with standing wave fluorescence

microscopy were immediately evident in our early work.

First, the use of total reflection in the illuminator was

convenient, but precluded the use of high-aperture

immersion lenses. Second, as described, the standing

wave data set consisted of a large number of images for

each specimen focal plane position, since both θ and Φ

could be varied. This made the method impractically

slow. The present methods and apparatus of excitation

field synthesis reduce the data set to one or two images

per focal plane, by multiplexing different standing-wave

fields.

With the beams counter propagating on axis ($\theta =$

0°), and an excitation wavelength of 514.5 nm, the node

spacing in the specimen was determined to be equal to

$\lambda/2n$ in both the mirror and crossed beam systems. In the

first case, mirror movement of 0.17 μm corresponds to a

shift of one fringe. In the two beam system, external

mirror movement of 0.514 μm had the same effect.

- 27 -

The standing-wave microscope was used to view cytoskeletal actin fibers in fixed 3T3 fibroblast cells. Cells were grown at a low density on 40mm cover glasses, fixed permeabilized, stained for F-actin with rhodamine-phalloidin, and mounted in a thin film of medium with a second cover glass on top. In the mirror system the specimens were oil-immersed to both the objective and the mirror. In the two-beam system, oil-immersion lenses were used on both sides of the specimen. With the standing-wave field adjusted for maximum fringe flatness, the laminar pattern of fluorescence excited in the specimen could be easily seen as contour-like rings resulting from the intersection of the planar antinodes with the mound-like volume occupied by the cytoskeleton. This was visible in even highly-flattened cells in which the cytoskeleton was entirely in sharp focus. By shifting the fringe position, different sets of fibers could alternatively be made to fluoresce. In certain cases, this type of contrast reversal was caused by reflector movement corresponding to a fringe displacement of 0.04-0.05 μm. From the direction of fringe displacement, and known orientation of the specimen, the axial order of distinguishable features could be inferred.

A comparison of standing wave to uniform excitation was made by blocking one path in the

- 28 -

microscope immediately after the beam splitter. In this
case, cytosketetal fibers in all parts of the specimen
fluoresce, and refocusing affected nearly all
distinguishable features equally, imparting little 3d
information. With the interference restored, regions of
the cell coincident with antinodes become approximately
4X brighter and nodal regions nonemissive. An image of
the same cell by confocal scanning fluorescence
microscopy showed less discrimination between sets of
actin fibers that show up in different standing wave
images.

Although we have described and illustrated
certain present preferred embodiments of our method and
apparatus for field synthesis and optical subsectioning
for standing wave microscopy, it should be understood
that our invention is not limited thereto, but may be
variously embodied within the scope of the following
claims.

- 29 -

CLAIMS:

1.   An improved optical microscope for observing
a luminescent specimen placed on a stage, such optical
microscope being of the type having a light source, a
lens which defines a focal plane which is a fixed
distance from the lens, a movable stage for holding the
specimen in the focal plane and means for producing a
standing wave field through the specimen to excite
fluorescence or phosphorescence in the specimen wherein
the improvement comprises:

   a)   a means for exciting the specimen by a
        time-multiplexed series of standing wave
        fields; and

   b)   means for recording an image of the
        specimen when the specimen is so excited.

2.   The improved optical microscope of claim 1
wherein the light source is a laser.

3.   The improved optical microscope of claim 1
wherein the specimen is between two microscope lenses and
also comprising a beam splitter and lens system, and
wherein the light source is positioned to create two
coherent light beams one beam passing through each
microscope lens and the beams crossing in the specimen.

- 30 -

4.   The improved optical microscope of claim 3 wherein the lens system includes a movable scan mirror to sweep the beams through the specimen so that the beams cross at equal angles.

5.   The improved optical microscope of claim 1 also comprising a mirror positioned to direct a light beam from the light source and a piezoelectric motor attached to the mirror.

6.   The improved optical microscope of claim 1 wherein the means for recording images is an electronic camera.

7.   The improved optical microscope of claim 1 also comprising:

    a)   a means for incrementally moving the
         specimen so that after each incremental
         movement the focal plane will pass through
         one of several parallel cross-sections
         through the specimen;

    b)   means for recording images of the specimen
         when the specimen is excited and at selected
         incremental positions; and

- 31 -

c)   an image processor for processing and

combining the images to produce at least one

combined image of the specimen.

8.   The improved optical microscope of claim 7

wherein the means for combining the images is comprised

of means for digitizing each image, memory for storing

digitized images, a processing unit for processing and

combining selected digitized images and display means for

displaying combined images.

9.   The improved optical microscope of claim 8

wherein the display means is one of a cathode ray tube

and film.

10.   The improved optical microscope of claim 1

also comprising a mirror apposed to the specimen.

11.   The improved optical microscope of claim 1

also comprising a phase conjugator apposed to the

specimen.

12.   The improved optical microscope of claim 1

wherein the light source is an incoherent light source

and also comprising a polarizer positioned to s-polarize

- 32 -

light beams emitted from the light source, a collimator and a wavelength filter to produce light of sufficient coherence for the method.

13.   An improved optical microscope for observing a luminescent specimen placed on a stage, such optical microscope being of the type having a lens which defines a focal plane which is a fixed distance from the lens, a movable stage for holding the specimen in the focal plane and a light source which emits light beams which strike the specimen at incidence angles for producing a standing wave field having a series of nodal planes through the specimen to excite fluorescence or phosphorescence in the specimen wherein the improvement comprises a means for moving a selected nodal plane without moving the specimen to null fluorescence from neighboring strata.

14.   A method employing a luminescence optical microscope having an optical axis for obtaining a three dimensional representation of an object comprising:

     a)   labeling the object with fluorescent or phosphorescent material;

     b)   disposing the object at the optical axis of the microscope;

c)   exciting the specimen by establishing a time

multiplexed series of standing wave fields;

and

d)   recording an image of the specimen.

15.   The method of claim 14 wherein the specimen
is excited by generating a continuous series of standing
wave fields that differ in node spacings and all standing
wave fields have an antinode at a focal plane of the
microscope.

16.   The method of claim 14 wherein the specimen
is excited by generating a continuous series of standing
wave fields that differ in node spacings and all standing
wave fields have a node at a focal plane of the
microscope.

17.   The method of claim 16 also comprising the
steps of

a)   exciting the specimen by generating a
continuous series of standing wave fields
that differ in node spacings and all
standing wave fields have an antinode at the
focal plane of the microscope;

b)   recording a second image of the specimen;
and

- 34 -

c) combining the image and the second image to

    create a combined image of the specimen.

18. The method of claim 17 wherein the combined
image is formed by subtracting the image from the second
image.

19. The method of claim 14 wherein the
fluorescent or phosphorescent material responds to light
of specific wave lengths and wherein the microscope has a
light source which emits only that wave length.

20. The method of claim 14 wherein the light
source is an incoherent light source and also comprising
the steps of collimating, s-polarizing and filtering
light beams emitted from the light source.

21. The method of claim 20 wherein the
microscope has a white light source which emits a light
beam and also comprising:

        a) filtering the light beam to permit only
            those wave lengths of light to pass which
            excite the fluorescent or phosphorescent
            material; and

        b) collimating the filtered light beam.

22. The method of claim 14 also comprising:

a) incrementally moving the specimen so that after each incremental movement the focal plane will pass through one of several parallel cross sections through the specimen;

b) establishing a time multiplexed series of standing wave fields for each incremental position of the specimen; and

c) recording at least one image of the specimen for each incremental position.

23. The method of claim 22 also comprising combining selected images for each incremental position of the specimen into a single combined image.

24. The method of claim 14 also comprising enhancing the image by use of an image processor.

25. The method of claim 14 wherein the image is recorded on film.

26. The method of claim 14 wherein the standing wave field has a selected nodal plane and is established by a light beam which is reflected from a mirror and also comprising the step of moving the mirror to cause an axial shift of the selected nodal plane through the specimen.

- 36 -

27. The method of claim 14 wherein the standing wave fields are created using the rteps comprising:

a) generating a light beam from a light source;

b) expanding the light beam; and

c) passing the light beam through a prism splitter to amplitude divide the beam so that a low divergence beam enters the specimen independently from opposite sides of the specimen.

28. A method employing a luminescence optical microscope for obtaining a three dimensional representation of an object comprising:

a) labeling said object with fluorescent or phosphorescent material;

b) disposing said object at the optical axis of said microscope;

c) exciting the specimen by establishing a first standing wave field having a nodal plane coincident with a stratum through the specimen;

d) recording a first image of the specimen;

e) moving the nodal plane a selected distance to null any fluorescence from a neighboring stratum;

f) recording a second image of the specimen; and

g) combining the first image and the second image.

29.   The method of claim 28 wherein the first image and the second image are combined by image processing.

30.   The method of claim 28 also comprising:

a)   moving the nodal plane a desired number of times and over a selected distance to null any fluorescence from a neighboring stratum;

b)   recording at least one subsequent image of the specimen after moving the nodal plane; and

c)   combining at least two images of the specimen selected from the group of images comprising the first image, the second image and subsequent images.
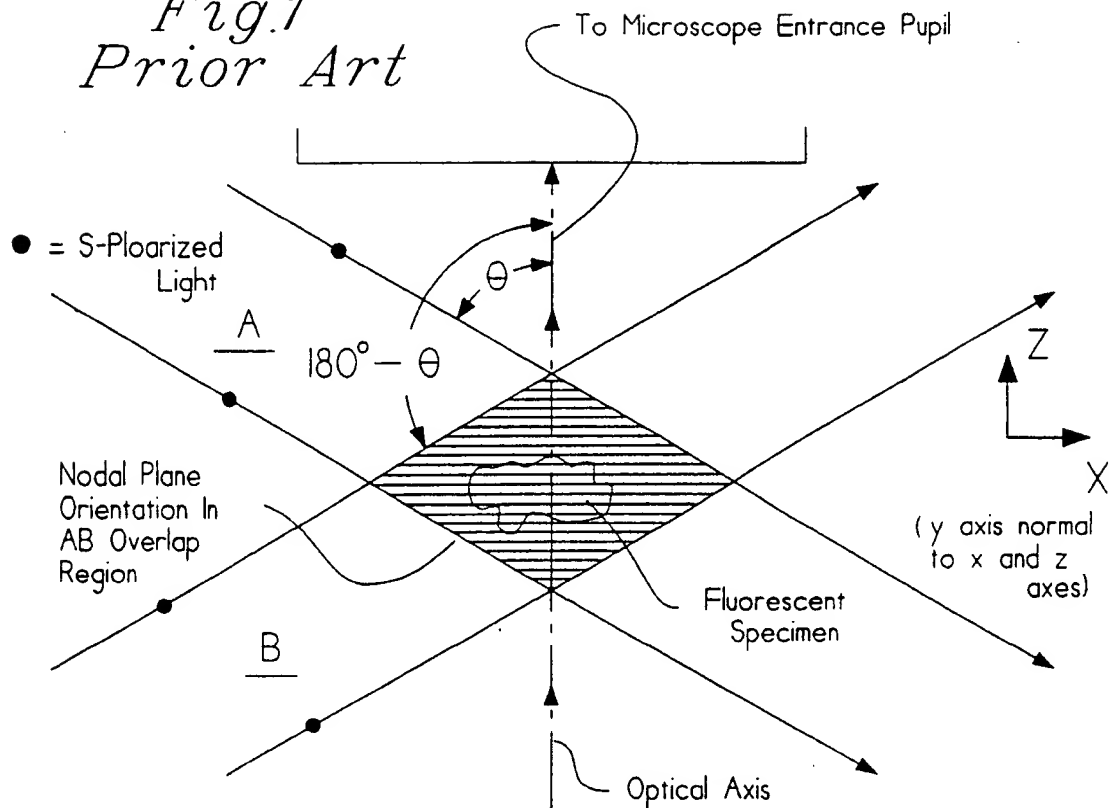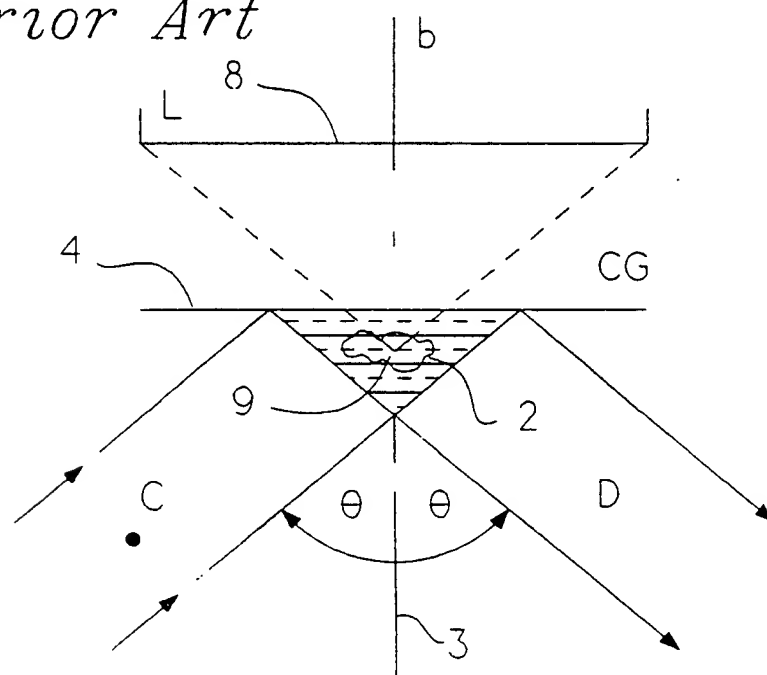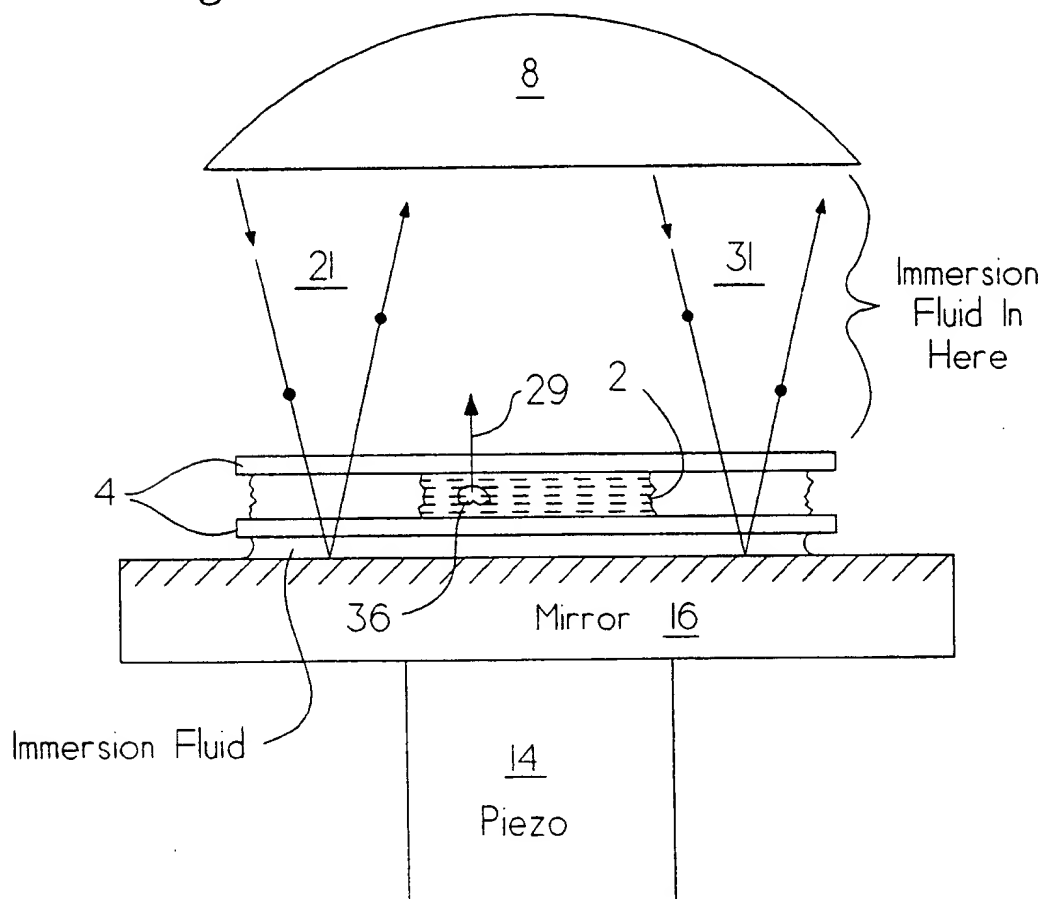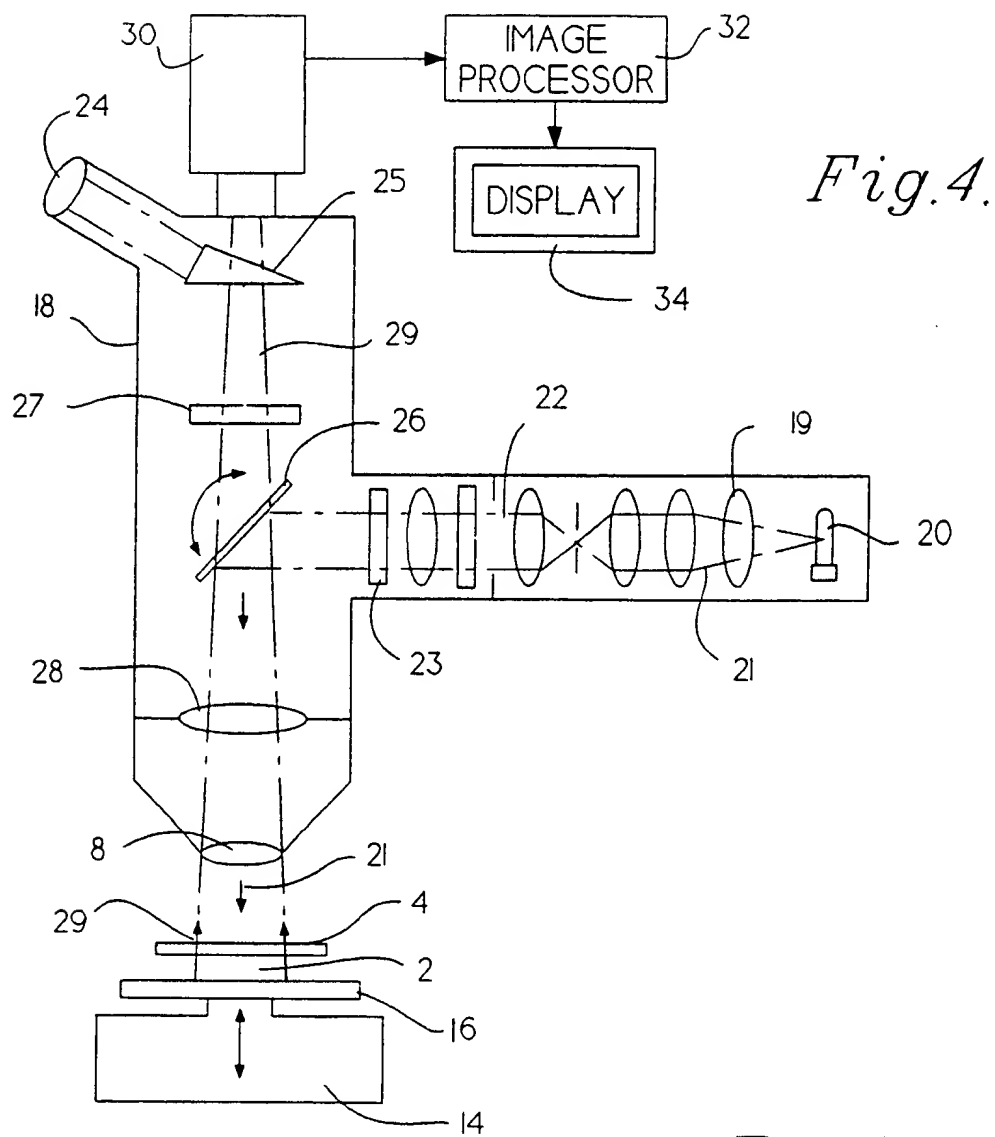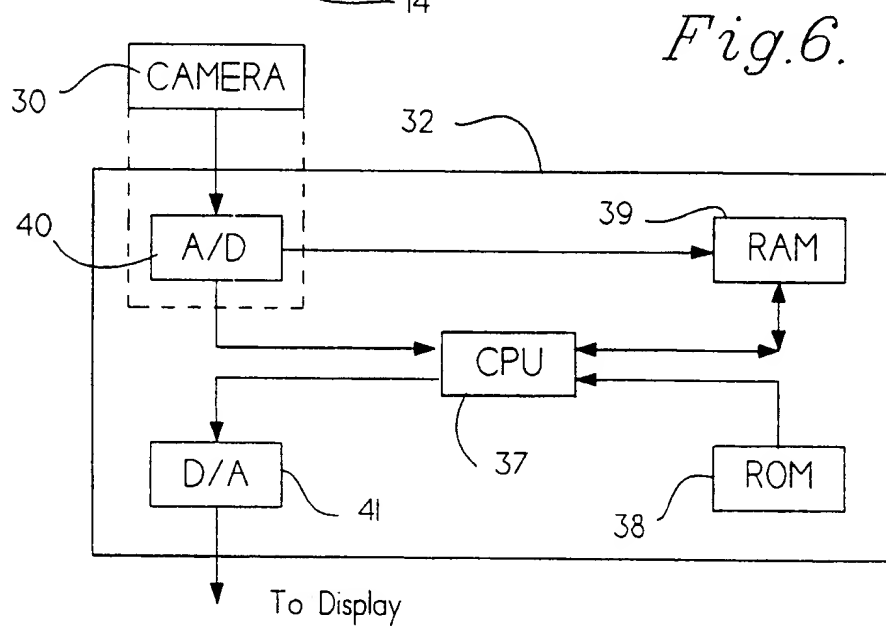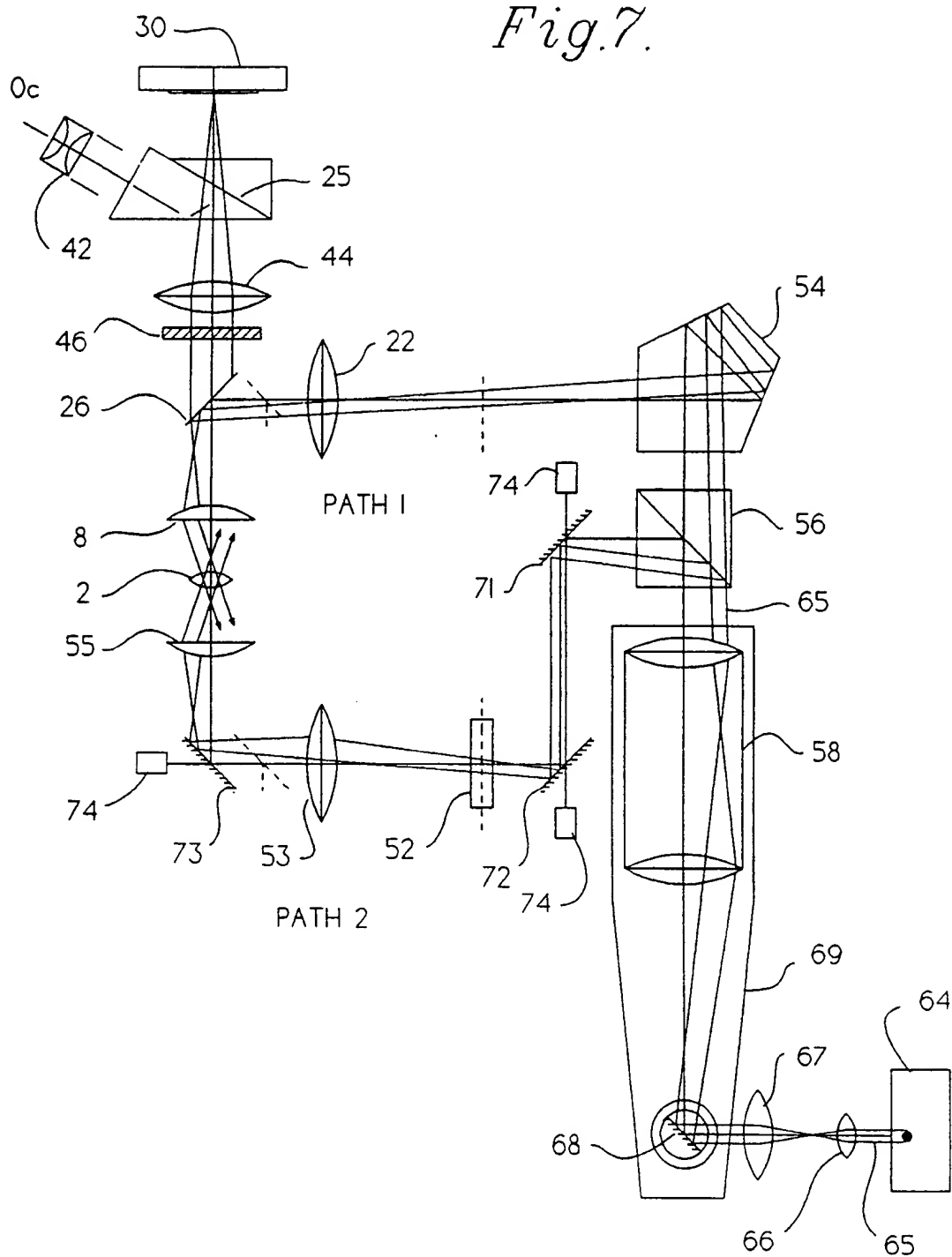
Fig.1
Prior Art

To Microscope Entrance Pupil
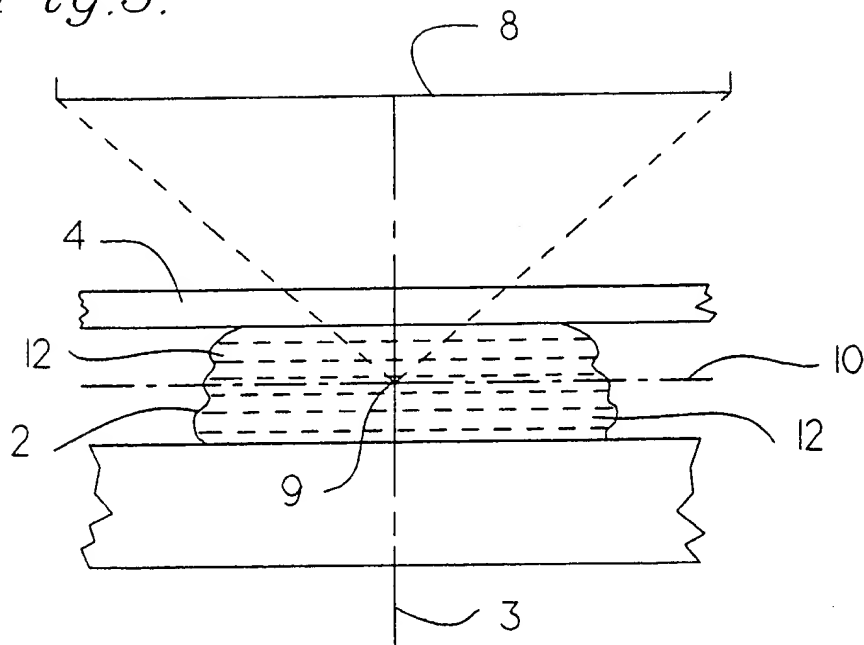
● = S-Ploarized Light

A

θ

180° − θ

Nodal Plane Orientation In AB Overlap Region

B

Z

X

(y axis normal to x and z axes)

Fluorescent Specimen

Optical Axis

Fig.2.
Prior Art

b

L   8

4   CG

9   2

C   D

θ   θ

3

Fig.3.

Fig.5.

Immersion Fluid In Here

Mirror 16

Immersion Fluid

14 Piezo

Fig.4.

Fig.6.

To Display

Fig.7.

Fig.8.

NODAL

ANTINODAL

DIFFERENCE
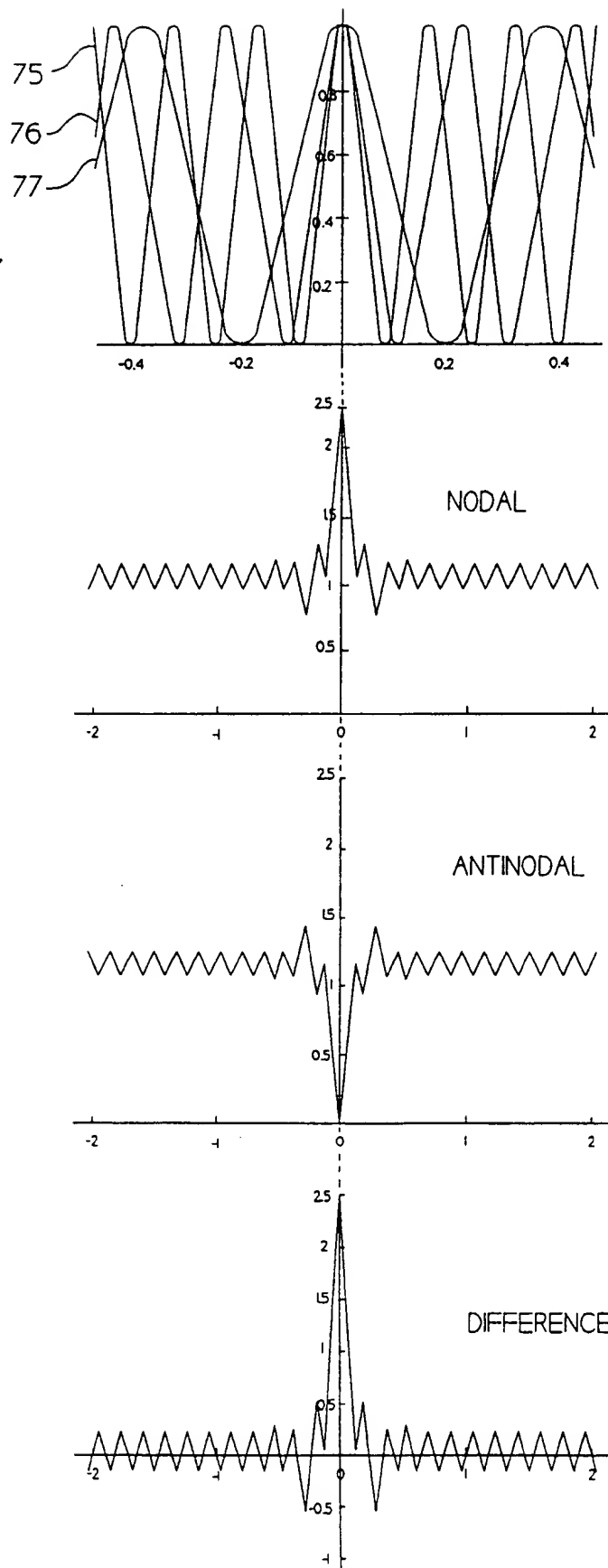
Fig.9.
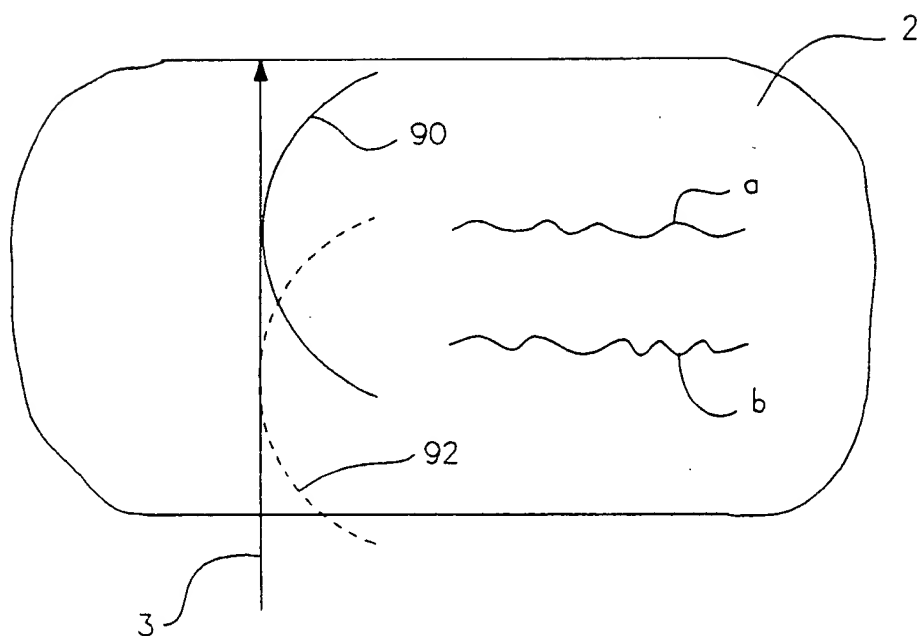
A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   G02B21/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   G02B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 621 911 (LANNI ET AL) 11 November 1986	13,28-30
A	see the whole document cited in the application	1,2,6, 10,14, 19,20,24

A	PROCEEDINGS OF THE SPIE: LOS ALAMOS CONFERENCE ON OPTICS, vol.190, 23 May 1979, LOS ALAMOS, NM, US pages 44 - 51 TEMPLE 'Improved Dark-field-like Surface Inspection Technique using Total Internal Reflection' see the whole document	1,2,10, 13,14, 28,30

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[X] Further documents are listed in the continuation of box C.	[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 June 1994	15. 06. 94

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Ward, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SOVIET PATENTS ABSTRACTS Section EI, Week 9218, Derwent Publications Ltd., London, GB; Class S03, Page 4, AN 92148427 & SU,A,1 374 922 (AS SIBE BIO-PHYS) 30 July 1991 see abstract; figure ---	1,13,14, 28
A	APPLIED OPTICS., vol.20, no.15, 1 August 1981, NEW YORK US pages 2656 - 2664 TEMPLE 'Total Internal Reflection Microscopy: A Surface Inspection Technique' see the whole document -----	1,13,14, 28

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4621911	11-11-86	NONE	